**AssayMax**™

# Rat ANP ELISA Kit

Assaypro LLC
3400 Harry S Truman Blvd
St. Charles, MO 63301
T (636) 447-9175
F (636) 395-7419
www.assaypro.com

For any questions regarding troubleshooting or performing the assay, please contact our support team at support@assaypro.com.

Thank you for choosing Assaypro.

## Assay Summary

**Step 1**. Add 50 µl of Standard or Sample per well.
Incubate 2 hours.

**Step 2.** Wash, then add 50 µl of Biotinylated Antibody per well.
Incubate 2 hours.

**Step 3**. Wash, then add 50 µl of SP Conjugate per well.
Incubate 30 minutes.

**Step 4.** Wash, then add 50 µl of Chromogen Substrate per well.
Incubate 10 minutes.

**Step 5.** Add 50 µl of Stop Solution per well.
Read at 450 nm immediately.

## Symbol Key

Consult instructions for use.

# Assay Template

| | A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | |
| 2 | | | | | | | | |
| 3 | | | | | | | | |
| 4 | | | | | | | | |
| 5 | | | | | | | | |
| 6 | | | | | | | | |
| 7 | | | | | | | | |
| 8 | | | | | | | | |
| 9 | | | | | | | | |
| 10 | | | | | | | | |
| 11 | | | | | | | | |
| 12 | | | | | | | | |

# Rat ANP ELISA Kit

Catalog No. ERA7010-1

***Sample insert for reference use only***

## Introduction

Atrial natriuretic peptide (ANP), a 28 amino acid polypeptide, is mainly secreted from the atrium of the heart where it is stored in secretory granules as a 136 amino acid pro-hormone (1). Upon its secretion, which is induced by increases in atrial pressure and stretch, the pro-hormone is processed by a serine protease to the active 28 amino acids peptide. The peptide binds with high affinity to the membrane receptor guanylate cyclase GC-A, leading to increased intracellular cGMP levels (2).

## Principle of the Assay

The AssayMax Rat ANP ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for detection of rat ANP in **plasma, serum, tissue extract, and cell culture samples**. This assay employs a quantitative **sandwich enzyme immunoassay** technique that measures rat ANP in less than 5 hours. A polyclonal antibody specific for rat ANP has been pre-coated onto a 96-well microplate with removable strips. The rat ANP in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for ANP, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

## Caution and Warning

- This product is for **Research Use Only** and is Not For Use In Diagnostic Procedures.
- Prepare all reagents (working diluent buffer, wash buffer, standard, biotinylated antibody, and SP conjugate) as instructed, prior to running the assay.
- Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this insert. However, the user should determine the optimal dilution factor.
- Spin down the SP conjugate vial and the biotinylated antibody vial before opening and using contents.
- The Stop Solution is an acidic solution.
- The kit should not be used beyond the expiration date.

## Reagents

- **Rat ANP Microplate:** A 96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against rat ANP.
- **Sealing Tapes:** Each kit contains 3 precut, pressure sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **Rat ANP Standard:** Rat ANP in a buffered protein base (20 ng, lyophilized).
- **Biotinylated Rat ANP Antibody (70x):** A 70-fold biotinylated polyclonal antibody against rat ANP (105 µl).
- **MIX Diluent Concentrate (10x):** A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x)**: A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate**: A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution**: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

## Storage Condition

- Upon arrival, immediately store components of the kit at recommended temperatures up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20°C.
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8°C.
- Unused microplate wells may be returned to the foil pouch with the desiccant packs and resealed. May be stored for up to 30 days in a vacuum desiccator.
- Diluent (1x) may be stored for up to 30 days at 2-8°C.
- Store Standard at 2-8°C before reconstituting with Diluent and at -20°C after reconstituting with Diluent.

## Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm.
- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl, and multiple channel).
- Deionized or distilled reagent grade water.

## Sample Collection, Preparation, and Storage

- **Plasma:** Collect plasma using a final concentration of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 3000 x *g* for 10

minutes and assay undiluted plasma for medium and high level of ANP. Samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles. For low level of ANP, please use ANP extraction protocol below.

**Low Level ANP Extraction Protocol**
Buffer A: 1% trifluoroacetic acid (TFA, HPLC Grade) in $H_2O$
Buffer B: 60% acetonitrile (HPLC Grade) in 1% TFA

1. Acidify the sample with equal amount of Buffer A (1 ml sample: 1 ml Buffer A). Mix and centrifuge samples at 6000 x *g* for 20 minutes at 4°C.
2. Pack an extraction column using 200 mg of C18 resin. Pre-equilibrate the column with 1 ml of Buffer B once and then with 3 ml of Buffer A three times.
3. Load the acidified plasma solution onto the pre-treated C18 column.
4. Slowly wash the column with 3 ml of Buffer A twice.
5. Elute the peptide slowly with 3 ml of Buffer B once and collect the eluant.
6. Evaporate and dry the eluant in a freeze dryer or use a suitable substitute method.
7. Keep the dried extract at -20°C and perform the assay as early as possible. Reconstitute the dried extract with 200 µl of MIX Diluent before the assay. Check sample pH with pH papers. If sample pH is below 6.5, neutralize the sample with 20 µl of 1M $NaH_2PO_4$. If the peptide value exceeds or does not fall in the range of detection, dilute or concentrate the sample accordingly.

- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x *g* for 10 minutes. Remove serum and perform the assay for medium and high level of ANP. Samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles. For low level of ANP, please use the extraction protocol as above.
- **Tissue:** Extract tissue samples with 0.1 M phosphate-buffered saline (pH7.4) containing 1% Triton X-100 and centrifuge at 14000 x *g* for 20 minutes. Collect the supernatant and measure the protein concentration. Freeze remaining extract at -20°C or below.
- **Cell Culture Supernatants:** Centrifuge cell culture media at 3000 x *g* for 10 minutes to remove debris. Collect supernatants and assay. Store samples at -20°C or below. Avoid repeated freeze-thaw cycles.

## Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.

- **MIX Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the MIX Diluent Concentrate 1:10 with reagent grade water. Store for up to 30 days at 2-8°C.
- **Rat ANP Standard:** Reconstitute the 20 ng of Rat ANP Standard with 5 ml of MIX Diluent to generate a 4 ng/ml standard stock solution. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the standard stock solution (4 ng/ml) 1:2 with MIX Diluent to generate 2, 1, 0.5, and 0.25 ng/ml solutions. MIX Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C and used within 30 days.

| Standard Point | Dilution | [Rat ANP] (ng/ml) |
|---|---|---|
| P1 | 1 part Standard (4 ng/ml) | 4.000 |
| P2 | 1 part P1 + 1 part MIX Diluent | 2.000 |
| P3 | 1 part P2 + 1 part MIX Diluent | 1.000 |
| P4 | 1 part P3 + 1 part MIX Diluent | 0.500 |
| P5 | 1 part P4 + 1 part MIX Diluent | 0.250 |
| P6 | MIX Diluent | 0.000 |

- **Biotinylated Rat ANP Antibody (70x):** Spin down the biotinylated antibody briefly and dilute the desired amount of the antibody 1:70 with MIX Diluent. Any remaining solution should be frozen at -20°C.
- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with MIX Diluent. Any remaining solution should be frozen at -20°C.

## Assay Procedure

- Prepare all reagents, standard solutions, and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-25°C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccants inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Rat ANP Standard or sample per well. Cover wells with a sealing tape and incubate for 2 hours. Start the timer after the last addition.

- Wash five times with 200 μl of Wash Buffer manually. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a machine, wash six times with 300 μl of Wash Buffer and then invert the plate, decanting the contents; hit 4-5 times on absorbent material to completely remove the liquid.
- Add 50 μl of Biotinylated Rat ANP Antibody to each well and incubate for 2 hours.
- Wash the microplate as described above.
- Add 50 μl of Streptavidin-Peroxidase Conjugate per well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.
- Add 50 μl of Chromogen Substrate per well and incubate for 10 minutes or till the optimal color density develops. Gently tap the plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 μl of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

## Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a standard curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

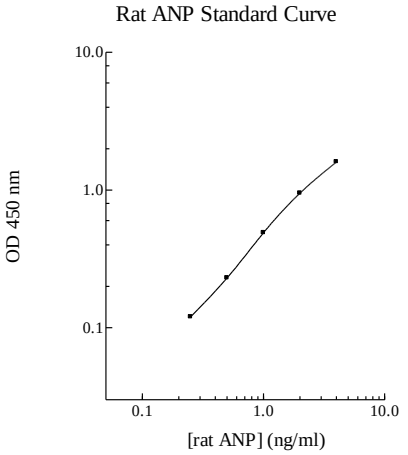
## Typical Data

- The typical data is provided for reference only. Individual laboratory means may vary from the values listed. Variations between laboratories may be caused by technique differences.

| Standard Point | ng/ml | OD | Average OD |
|---|---|---|---|
| P1 | 4.000 | 1.855<br>1.874 | 1.864 |
| P2 | 2.000 | 1.499<br>1.467 | 1.483 |
| P3 | 1.000 | 0.898<br>0.894 | 0.896 |
| P4 | 0.500 | 0.364<br>0.382 | 0.373 |
| P5 | 0.250 | 0.214<br>0.220 | 0.217 |
| P6 | 0.000 | 0.128<br>0.114 | 0.121 |
| **Sample: Normal,<br>Rat Sodium Citrate Plasma (1x)** | | 0.945<br>0.927 | 0.936 |

## Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.




## Performance Characteristics

- The minimum detectable dose of rat ANP as calculated by 2SD from the mean of a zero standard was established to be 0.09 ng/ml.
- Intra-assay precision was determined by testing replicates of three plasma samples in one assay.
- Inter-assay precision was determined by testing three plasma samples in twenty assays.

| | Intra-Assay Precision | | | Inter-Assay Precision | | |
|---|---|---|---|---|---|---|
| Sample | 1 | 2 | 3 | 1 | 2 | 3 |
| n | 20 | 20 | 20 | 20 | 20 | 20 |
| CV (%) | 2.1% | 2.6% | 2.2% | 7.5% | 8.1% | 7.9% |
| Average CV (%) | 2.3% | | | 7.8% | | |

## Recovery

| | |
|---|---|
| Standard Added Value | 0.3 – 1.3 ng/ml |
| Recovery % | 86 – 108% |
| **Average Recovery %** | 101% |

## Cross-Reactivity

| Species | Cross Reactivity (%) |
|---|---|
| Canine | <40% |
| Bovine | None |
| Monkey | <40% |
| Mouse | <40% |
| Rat | 100% |
| Swine | 100% |
| Human | <40% |

## Troubleshooting

| Issue | Causes | Course of Action |
|---|---|---|
| **Low Precision** | Use of expired components | • Check the expiration date listed before use.<br>• Do not interchange components from different lots. |
| | Improper wash step | • Check that the correct wash buffer is being used.<br>• Check that all wells are dry after aspiration.<br>• Check that the microplate washer is dispensing properly.<br>• If washing by pipette, check for proper pipetting technique. |
| | Splashing of reagents while loading wells | • Pipette properly in a controlled and careful manner. |
| | Inconsistent volumes loaded into wells | • Pipette properly in a controlled and careful manner.<br>• Check pipette calibration.<br>• Check pipette for proper performance. |
| | Insufficient mixing of reagent dilutions | • Thoroughly agitate the lyophilized components after reconstitution.<br>• Thoroughly mix dilutions. |
| | Improperly sealed microplate | • Check the microplate pouch for proper sealing.<br>• Check that the microplate pouch has no punctures.<br>• Check that three desiccants are inside the microplate pouch prior to sealing. |

| | | |
|---|---|---|
| **Unexpectedly Low or High Signal Intensity** | Microplate was left unattended between steps | • Each step of the procedure should be performed uninterrupted. |
| | Omission of step | • Consult the provided procedure for complete list of steps. |
| | Steps performed in incorrect order | • Consult the provided procedure for the correct order. |
| | Insufficient amount of reagents added to wells | • Check pipette calibration.<br>• Check pipette for proper performance. |
| | Wash step was skipped | • Consult the provided procedure for all wash steps. |
| | Improper wash buffer | • Check that the correct wash buffer is being used. |
| | Improper reagent preparation | • Consult reagent preparation section for the correct dilutions of all reagents. |
| | Insufficient or prolonged incubation periods | • Consult the provided procedure for correct incubation time. |
| **Deficient Standard Curve Fit** | Non-optimal sample dilution | • Sandwich ELISA: If samples generate OD values higher than the highest standard point (P1), dilute samples further and repeat the assay.<br>• Competitive ELISA: If samples generate OD values lower than the highest standard point (P1), dilute samples further and repeat the assay.<br>• User should determine the optimal dilution factor for samples. |
| | Contamination of reagents | • A new tip must be used for each addition of different samples or reagents during the assay procedure. |
| | Contents of wells evaporate | • Verify that the sealing film is firmly in place before placing the assay in the incubator or at room temperature. |
| | Improper pipetting | • Pipette properly in a controlled and careful manner.<br>• Check pipette calibration.<br>• Check pipette for proper performance. |
| | Insufficient mixing of reagent dilutions | • Thoroughly agitate the lyophilized components after reconstitution.<br>• Thoroughly mix dilutions. |

## References


(1) Atlas SA *et al.* (1992) p.1577-673

(2) Leitman, DC *et al.* (1988) *J. Biol. Chem.* 263:3720-8


Version 2.3R